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We have isolated a prostate-specific gene, *NKX3.1*, that maps to chromosome 8p21, a common region for loss of heterozygosity in human prostate cancer. *NKX3.1* is a homeobox gene that is expressed at high levels in adult human prostate. Deletion of *NKX3.1* in the mouse has suggested that *NKX3.1* has a suppressor effect on prostatic epithelial cell growth, and that haploinsufficiency is sufficient to produce epithelial hyperplasia. Inconsistent with the interpretation that *NKX3.1* is a classical tumor suppressor gene, the coding region of *NKX3.1* is not mutated in human prostate cancer. This is the first annual report of a project that seeks to determine the significance of NKX3.1 in prostate cancer. We have shown that a genetic polymorphism that changes DNA-binding properties of the NKX3.1 protein is present in approximately 10% of the human population and is not strongly associated with prostate cancer risk. We continue to investigate whether the R52C NKX3.1 polymorphism influences the development of prostatic hyperplasia. We have derived a specific antibody to human NKX3.1 and are investigating control of expression of NKX3.1 in human prostate cancer.

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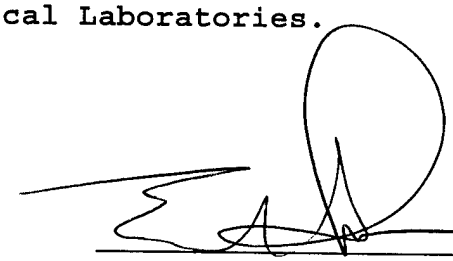

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Introduction

We have isolated a prostate-specific gene, *NKX3.1*, that maps to chromosome 8p21, a common region for loss of heterozygosity in human prostate cancer. *NKX3.1* is a homeobox gene that is expressed at high levels in adult human prostate, at very low levels in testis and in no other tissues (1). Expression of *NKX3.1* is androgen-regulated and preliminary data indicates that *NKX3.1* regulates androgen receptor expression. Moreover, mice either heterozygous or homozygous for deletion of *NKX3.1* had prostatic hyperplasia and an elevated growth fraction of prostatic epithelial cells (2). This implies that *NKX3.1* has a suppressor effect on prostatic epithelial cell growth, and that haploinsufficiency is sufficient to produce epithelial hyperplasia. Inconsistent with the interpretation that *NKX3.1* is a classical tumor suppressor gene, the coding region of *NKX3.1* is not mutated in human prostate cancer (3). However, disruption of a single allele or diminished expression of *NKX3.1* may occur in human prostate cancer and contribute to prostate neoplasia. For this reason we are characterizing the promoter region of *NKX3.1* to search for mutations and study methylation patterns that may influence expression in prostate cancer. We are also investigating levels of NKX3.1 expression in cancer cells to see if expression of NKX3.1 is diminished in prostate cancer. We have also found a polymorphism in *NKX3.1* that changes amino acid 52 from arginine to cysteine (R52C). The polymorphism disrupts a consensus phosphorylation sequence and abrogates dependence of DNA binding on phosphorylation in vitro. This provides a basis for asking about the role of R52C in prostate cancer risk.

Body

The approved Statement of Work had five aims. This Statement of Work anticipated a budget of $125,000. The project was funded at 75% of the requested support level. This progress report is organized under headings of each of the aims of the original Statement of Work.

Aim 1: Mutational analysis of the *NKX3.1* promoter region

The original human genomic clone of *NKX3.1* contained 1.3 Kb of the 5' untranslated region. We found this region to have little or no transcriptional activity when placed upstream from a luciferase reporter construct. For this reason we have pursued two strategies to identify the critical promoter elements of *NKX3.1*. We are in the process of cloning the region of DNA upstream from exon 1 of *NKX3.1*. Using a fragment taken from the extreme 5' end of the human genomic clone, we are in the process of screening a phage λ human genomic library to characterize and sequence 10-20 Kb upstream from *NKX3.1*.

We also want to determine whether the first intron plays a role in regulating transcription. To determine if there are promoter or enhancer sequences in this region we have isolated the intron by polymerase chain reaction and confirmed its identity by nucleotide sequencing. We then subcloned the intron in both forward and reverse orientation upstream and downstream from an SV40 promoter in a luciferase reporter plasmid. These constructs are complete and will be used in reporter gene assays to determine if intron 1 has sequences that can affect transcription.

Aim 2: *NKX3.1* R52C polymorphism in racial groups and in prostate cancer patients and controls

Using the Taqman assay described in our proposal to determine *NKX3.1* genotype, we have been working with two cohorts of DNA to assess the racial distribution of the R52C polymorphism of *NKX3.1* and to determine whether the polymorphism has an effect on prostate cancer risk. We have analyzed approximately 750 samples from prostate cancer cases and age-matched controls from the Physicians Health Study. The data is shown in Table 1. The relative risk = 1.55 (95% CI = 0.93-2.58). The data are not statistically significant at this point. We have received and are testing 400 additional samples to increase the power of this cohort and determine whether *NKX3.1* R52C has a low level of influence on prostate cancer risk.

Table 1 – *NKX3.1* Genotype in Physicians Health Study Participants

	Allele		Total
	CC	CT or TT	
Prostate Cancer	330	42 (11.3%)	372
Controls	346	30 (7.98%)	376

Since the Physicians Health Study cohort was composed mostly of Caucasians, we have used a smaller cohort from Richard Hayes, PhD, of the National Cancer Institute, to determine the racial distribution of the *NKX3.1* R52C polymorphism. The data are shown in Table 2. Differences in Table 2 are not statistically significant. A chi-square test (two-sided) yielded P=0.13 If we hypothesized that Caucasians had a higher percent of R52C than African Americans (i.e. hypothesis was one-sided), the difference between 13% gene B for Whites and 8.7% gene B for blacks wouldn't quite achieve statistical significance at the .05 level (P=.065).

Table 2 – Racial Distribution of the *NKX3.1* R52C Polymorphism

	Allele		Total
	CC	CT or TT	
Caucasian	222	33 (12.9%)	255
African American	198	19 (8.6%)	217

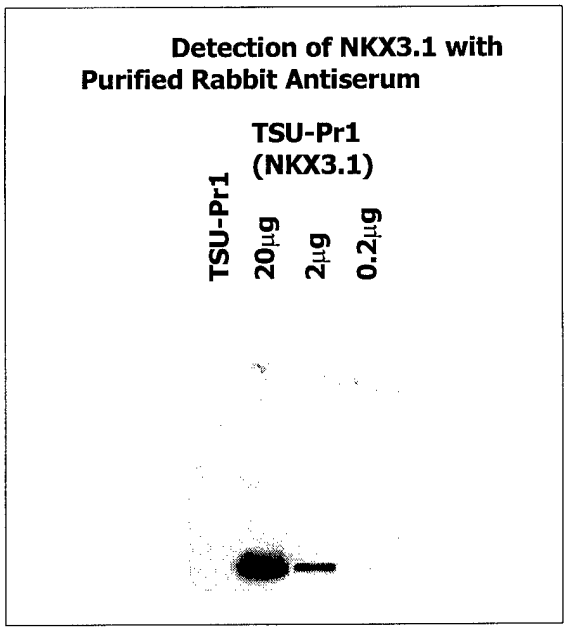
Further genotyping experiments will explore other hypotheses. Based on the mouse deletion mutant data we will ask whether *NKX3.1* R52C is associated with prostatic enlargement and hyperplasia. We will study DNA from a cohort of patients at Johns Hopkins University with familial prostatic hyperplasia. We will also study approximately 600 participants of the Prostate, Lung, Colon and Ovarian Cancer Screening Trial (PLCO) to compare *NKX3.1* genotypes between men with very large and very small prostate glands.

Aim 3: Immunohistochemical detection of NKX3.1 protein in prostate cancer tissues.

We have tested rabbit antisera to murine NKX3.1 peptides for reactivity against cultured cells expressing NKX3.1 and against human tissue sections. These antisera have not proved to be useful for immunohistochemical detection of NKX3.1 in human tissues even though they detect the human protein on western blots. We have therefore proceeded to make recombinant human NKX3.1 protein to generate

our own antibody. Recombinant protein was made using a plasmid expression vector that creates a fusion protein between NKX3.1 and bacterial mannose binding protein (MBP). The fusion protein can be purified on an amylose column and cleaved from MBP by digesting with a protease called genenase. Purified NKX3.1 was used with adjuvant to generate rabbit antisera. Serum from test bleeds have been absorbed with bacterial lysates and purified on columns with cyanogen bromide-bound NKX3.1. Purified antibody was used to detect NKX3.1 in transfected TSU-Pr1 prostate cancer cells is shown in Figure 1. The next step will be to test the antiserum on cultured cells and tissues prepared with different fixatives.

FIGURE 1

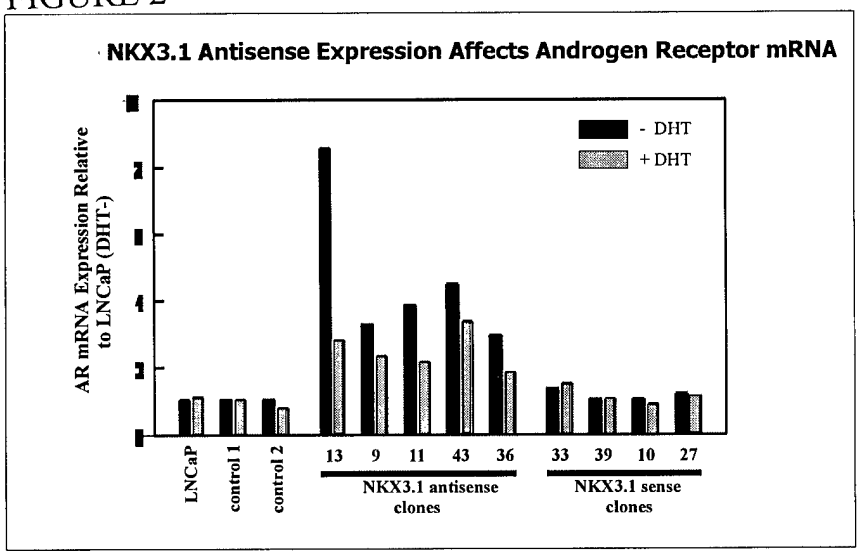


Aim 4: Identify the effect of *NKX3.1* expression on prostate cancer phenotype in cultured cells

We have transfected *NKX3.1* expression vectors into the LNCaP and TSU-Pr1 human prostate cancer cell lines. Since LNCaP cells express endogenous *NKX3.1*, we also transfected antisense constructs into these cells to down regulate the expression of NKX3.1. The expression of NKX3.1 in the TSU-Pr1 transfectants was confirmed by RNase protection analysis and by western blotting. We also confirmed that NKX3.1 expression in LNCaP cells transfected with *NKX3.1* antisense was somewhat diminished.

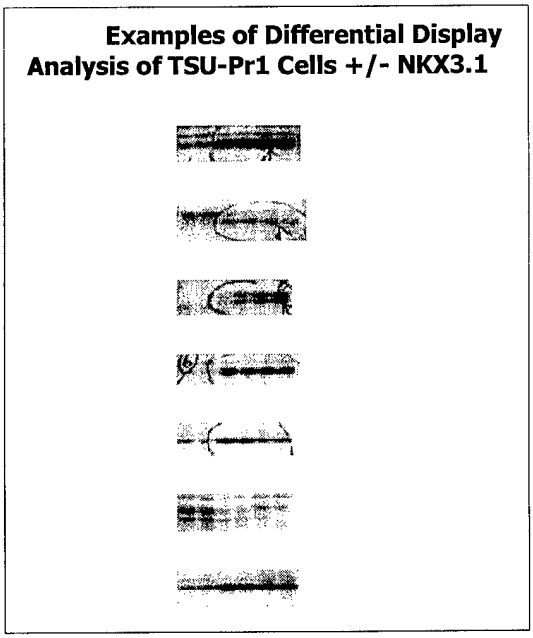
We saw no effect of NKX3.1 expression on in vitro cell growth or morphology of TSU-Pr1 cells. LNCaP cells showed no effects of either sense or antisense NKX3.1 constructs on in vitro cell growth, morphology or hormone-dependence. We found that NKX3.1 antisense expression had a positive effect on the expression of androgen receptor mRNA. AR mRNA was up regulated in 5 of 5 clones that expressed antisense *NKX3.1*, but AR mRNA not affected in either control transfectants or transfectants with the sense constructs (Figure 2). Moreover, addition of 10^{-9} M DHT to the cultures, which induces endogenous *NKX3.1* expression, down regulated AR message in the antisense transfectants. This result suggests that *NKX3.1* modulates expression of AR. Note that in the control cells DHT had no effect on AR mRNA.

FIGURE 2



In order to identify genes that may have had altered expression by NKX3.1 we performed differential display analysis using mRNA from TSU-Pr1 cells and from clones transfected with *NKX3.1*. Examples of gene products with altered expression are shown in Figure 3. The six lanes shown represent three sets of duplicate samples each generated by independent PCR reactions. The first two lanes are from TSU-Pr1 cells; the latter four lanes from the same cells transfected with *NKX3.1*. Note that there are both up and down regulated genes. Sequence analysis of these fragments has identified at least two interesting transcripts that may reflect phenotypic changes induced by NKX3.1. Two upregulated genes were lipocortin II and tubulin. Lipocortin II or annexin II belongs to a family of Ca2+- and phospholipid-binding proteins whose function is not clearly defined. Members of the annexin family have been implicated in membrane-related events along exocytotic and endocytotic pathways (4-7). Lipocortins were found to be expressed by prostatic cells and are present in prostate secretions (8,9). Expression of tubulin in NKX3.1-expressing cells is noteworthy because tubulin was also found to be activated by androgen in the androgen-sensitive tumor xenograft CWR22. Tubulin expression was also increased in androgen-independent derivatives of CWR22 (10). The activation of these two genes suggests that NKX3.1 may have differentiation-inducing properties in prostatic epithelial cells and may mediate some effects induced by androgen.

FIGURE 3



Aim 5: Determine if RT-PCR for *NKX3.1* can be applied to detect circulating prostate cancer cells

Expression of NKX3.1 is highly restricted in the mouse to prostatic lobes and bulbourethral gland (2). NKX3.1 expression is less tightly controlled in adult human tissues. There is a low level of expression in the testis and a faint signal on northern blots of peripheral blood mononuclear cell RNA. We tested whether NKX3.1 could be used in human blood samples to detect low levels of prostatic carcinoma cells. However, we found that with nested PCR reactions we could detect NKX3.1 sequences in the blood of normal men and women. We therefore have concluded that NKX3.1 will not be a useful marker for detection of circulating micrometastatic prostatic cells in patients with prostate cancer.

Key Research Accomplishments

- Determined frequency of *NKX3.1* R52C polymorphism in the population and in cancer patients
- Developed a rabbit antiserum to recombinant human NKX3.1
- Identified two NKX3.1-regulated genes, lipocortin II and tubulin
- Determined that *NKX3.1* is expressed in normal human peripheral blood cells

Reportable Outcomes

1. Funding applied for and received:

Genetic polymorphisms in prostate cancer
NIEHS ES-09888
PI – Edward Gelmann

2. Employment or research opportunities

David Steadman, PhD
Postdoctoral fellow received funding in 1999 from the US Army prostate cancer research program for studies of the biochemistry of NKX3.1 binding to DNA.

Conclusions

This is the first annual report of a project that seeks to determine the significance of NKX3.1 in prostate cancer. Recent work has shown that in the mouse, NKX3.1 is an important regulator of prostatic cell growth and differentiation. We are investigating control of expression of NKX3.1 in human prostate cancer. We have shown that a polymorphism in the gene, present in about 10% of the population, is not a major risk factor for prostate cancer. We continue to investigate whether the R52C NKX3.1 polymorphism influences the development of prostatic hyperplasia.

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Appendices

none